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11.0 PRACTICAL CONSIDERATIONS

The 3T3 and NHK NRU test methods are proposed as adjuncts, rather than replacements for, the *in vivo* acute oral toxicity assays. Data from these *in vitro* basal cytotoxicity test methods are used with a prediction model to estimate the rodent oral LD_{50} of the test chemical. This LD_{50} value is then used to determine the starting dose for subsequent *in vivo* acute oral toxicity assays. This section discusses practical issues involved in applying these two *in vitro* NRU cytotoxicity test methods to the prediction of starting doses for rodent acute systemic toxicity assays. Practical issues to consider for implementation of these cell culture test methods include the need for and availability of specialized equipment, training and expertise requirements, cost considerations, and time expenditure. Good Cell Culture Practice: ECVAM Good Cell Culture Practice Task Force Report 1 (Hartung et al. 2002) encourages the establishment of practices and principles that will reduce uncertainty in the development and application of *in vitro* test methods.

Good cell culture practices (in conjunction with good laboratory practices) are essential for all *in vitro* cytotoxicity testing and should be employed to ensure that data produced from the 3T3 and NHK NRU test methods are reproducible, reliable, credible, and acceptable.

11.1 Transferability of the 3T3 and NHK NRU Test Methods

Transferability of a test method is defined as the ability of a test method or procedure to be accurately and reliably performed in different, competent laboratories (ICCVAM 2003). Accuracy and reliability of these test methods are discussed in **Sections 6** and **7**, respectively.

Protocols for the 3T3 and NHK NRU test methods, solubility testing, and prequalification of keratinocyte growth medium have been optimized and are available on the ICCVAM/NICEATM website (http://iccvam.niehs.nih.gov/methods/invitro.htm). The protocols were designed with GLP-compliance in mind and can be easily implemented or adapted by scientists with the appropriate technical experience.

While *in vitro* and *in vivo* methods require some similar skills (e.g., preparation of solutions and test chemical doses, documentation), *in vitro* testing requires skills specific to cell culture systems (e.g., aseptic techniques, microscopic evaluation of cell cultures, propagation of cells in medium) but not to the maintenance, handling, or treatment of rodents.

11.1.1 Facilities and Major Fixed Equipment

The following lists of facility requirements, equipment and supplies, and training and expertise are common to most *in vitro* mammalian cell culture laboratories. Required equipment and supplies are also described in the NICEATM/ECVAM validation study 3T3 and NHK NRU test method protocols (**Appendices B and C**), the *Guidance Document* (ICCVAM 2001b, **Appendix D**) and Hartung et al. 2002.

Facility Requirements

The testing facility should provide structures and infrastructures necessary for operating a scientific laboratory (e.g., laboratory space, access to utilities, shipping/receiving department [for appropriate receipt and handling of cell culture materials], etc.). Each facility should provide:

- personnel that are competent in performing *in vitro* cytotoxicity assays under aseptic laboratory conditions
- adequate facilities, equipment, and supplies
- proper health and safety guidelines
- satisfactory quality assurance procedures

Each facility should conform to all appropriate statutes (i.e., local, state, provincial, federal, national, international) concerning safety guidelines (e.g., general workplace safety guidelines, chemical handling and disposal guidelines, biohazard guidelines, etc.). Hartung et al. 2002 provides recommended safety guidelines for working with potentially infectious materials (e.g., HIV, hepatitis B, hepatitis C) and human materials (e.g., cells, tissues, fluids).

The facility management should establish scientific guidelines and procedures, train and supervise professional and technical staff, and evaluate results and performance within their

discipline area relative to the testing requirements. Personnel should have mandatory training in basic cell culture practice, in specific procedures for specialized culture procedures, and in specific safety practices appropriate to the types of materials that may be used in the laboratory (Hartung et al. 2002). The management should maintain records of the qualifications, training and experience, and job descriptions for each professional and technical individual involved in the testing.

Cell Culture Laboratory

The testing facility should have a designated cell culture laboratory to ensure that *in vitro* cytotoxicity assays are performed under clean and proper aseptic conditions. The laboratory should be located such that through traffic is minimal to reduce possible disturbances that may compromise the cell culture assays. Room temperature of the laboratory should be regulated, monitored, and documented. Access to the laboratory and test chemicals should be restricted to appropriate personnel.

Major Equipment

Each testing facility should have at a minimum the following equipment:

- incubator (37ºC ± 1ºC, 90% ± 10% humidity, 5.0% ± 1% CO_2/air)
- laminar flow clean bench/cabinet (standard: "biological hazard")
- inverse phase contrast microscope
- 96-well plate spectrophotometric plate reader equipped with 540 nm ± 10 nm filter (if testing in 96-well plates)
- autoclave
- refrigerator
- freezer (-70ºC)
- liquid nitrogen
- cryogenic freezer/storage unit
- computer

Equipment maintenance and calibration should be routinely performed and documented as per GLP guidelines and testing facility procedures.

11.1.2 Availability of Other Necessary Equipment and Supplies

General Equipment

Each testing facility should have at a minimum the following equipment:

- centrifuge
- waterbath
- pipettors
- balance
- pH meter
- cell counting system
- water bath sonicator
- magnetic stirrer
- vortex mixer
- antistatic bar ionizer

Equipment maintenance and calibration should be routinely performed and documented as per GLP guidelines and testing facility procedures. These types of equipment are available from scientific and laboratory supply companies (e.g., Fisher Scientific, Thomas Scientific, etc.).

General Cell Culture Materials and Supplies

The following supplies are needed for the NRU test methods:

- tissue culture plasticware
- glassware
- sterile filtration systems
- culture medium and supplements
- serum
- balanced salt solutions
- NRU assay chemicals

Cell culture supplies are generally available through the major scientific and laboratory supply companies and through specialty companies (e.g., GIBCO, SIGMA-Aldrich, CAMBREX/Biowhittaker, Becton Dickinson, etc.). Compositions of culture media, supplements/additives, salt solutions, NRU assay chemicals and the volumes needed for the test methods should be defined. All culture vessels needed to assure proper cell propagation should be defined.

During this study, obtaining an adequate supply of NHK medium was problematic for FAL. Communication between the UK distributor and the laboratory was uneven and the SMT intervened on several occasions in an attempt to resolve the supply issue. This illustrates the need for additional sources of keratinocyte cell culture medium. Periodically, it was also difficult to obtain NHK medium and supplements that adequately supported keratinocyte growth similarly in all the laboratories. Although the purchased medium met the manufacturer's QA/QC standards, certain lots of the medium and supplements did not support the growth of NHK cells to the extent needed to meet the growth characteristics required by the test method protocol. This necessitated the need to incorporate an NHK medium prequalification protocol into the study. Prequalification of medium is recommended to avoid unnecessarily repeating studies.

Cell Cultures

3T3 Mouse Fibroblasts: BALB/c 3T3 cells, clone 31, can be obtained from national/international cell culture repositories (e.g., CCL-163, American Type Culture Collection [ATCC], Manassas, VA).

Normal Human Epidermal Keratinocytes (NHK): non-transformed keratinocyte cells from cryopreserved primary or secondary cells can be obtained from national/international cell culture repositories (e.g., CAMBREX Bio Science, 8830 Biggs Ford Road, Walkersville, MD) or isolated from donated tissue (using proper collection, preparation, and propagation techniques).

Obtaining adequate supplies of keratinocytes may be difficult since preparing a pool of cells depends on the availability of tissue donors. Procurement of a commercially available stock pool of cells and storing them indefinitely in a cryogenics freezer is recommended.

11.2 3T3 and NHK NRU Test Method Training Considerations

11.2.1 Required Training and Expertise

Hartung et al. 2002 recommends that scientists involved in *in vitro* testing should have training in basic cell culture aspects such as: sterile technique, handling culture media, feeding cultures, cell counting, subculture (trypsinization), detection and elimination of contamination, growth parameters, growth curves, viability assays, storage and freezing/thawing of cells. Additionally, training is encouraged for special culture procedures such as: primary cell and tissue cultures, toxicity testing, viability assays, cloning, transfection, expression cloning, cell transformation and immortalization, and virus propagation and isolation. Laboratory personnel should be trained in the application of GLP requirements (see **Section 8.1.1**).

Training and Expertise

In vitro NRU cytotoxicity test methods require personnel trained specifically in sterile tissue/cell culture techniques and general laboratory procedures. Performance of the test methods requires a relatively moderate degree of technical capability and a high degree of skill in monitoring and maintaining appropriate cell growth conditions, troubleshooting potential and real problems in culture systems, and interpreting and analyzing cytotoxicity data. Each individual engaged in the conduct of or responsible for the supervision of a study shall have education, training, and experience, or combination thereof, to enable that individual to perform the assigned duties. The NRU test methods do not require that personnel be trained to perform *in vivo* testing.

Specific Training and Expertise Needed for the In Vitro NRU Cytotoxicity Test Methods

Personnel involved in performing the *in vitro* NRU cytotoxicity test methods should be well experienced in general cell culture techniques and should be able to:

- work with cryogenic freezing apparatus
- pipette solutions with large volume pipettors and multi-channel pipettors
- establish cells in culture vessels under aseptic conditions and monitor growth; recognize normal and abnormal cell growth characteristics; document observations of cell cultures throughout all aspects of the cultures
- perform the *in vitro* assays by following the protocols to: grow the cells, treat the cells with test chemicals, perform the NRU assay, measure endpoints (i.e., optical density measurements), transfer data to electronic templates
- operate equipment necessary for maintaining cell culture laboratories (e.g., incubators, biohazard hoods, spectrophotometric microtiter plate readers)

General Laboratory Expertise Needed for the In Vitro NRU Cytotoxicity Test Methods

Personnel should also be able to perform and understand basic laboratory techniques and laboratory management:

- prepare cell culture solutions (e.g., culture medium, NRU solutions); measure pH; know proper storage conditions and maintain proper documentation
- prepare test chemicals for application to cell culture test plates; follow solubility protocols to adequately prepare test chemicals in solution; recognize solubility issues (e.g., insolubility nature of chemical, precipitation) and implement mechanical procedures for solubilizing the test chemicals
- monitor and control laboratory room conditions (e.g., temperature, humidity, lighting, traffic); maintain equipment at conditions essential to cell cultures (e.g., temperature, humidity, gas flow, calibrations)

Personnel Needed to Perform the In Vitro NRU Cytotoxicity Test Methods

- Study Director: the single point of study control; has the overall responsibility for the technical conduct of the testing (e.g., GLP adherence); determines test acceptance, provides SOPs, interprets and analyzes the data, documents testing aspects, and produces all written reports.
- Quality Assurance Officer: monitors the testing to assure conformance with GLP requirements; must be independent of the Study Director.

- Laboratory Technician(s): individuals trained in sterile tissue/cell culture techniques and general laboratory procedures and capable of performing the *in vitro* NRU cytotoxicity test methods in a GLP-manner.

11.2.2 Training Requirements to Demonstrate Proficiency

Laboratories set their own criteria for proficiency, but in general, personnel should be able to understand the protocol, carry out the protocol with guidance from an experienced supervisor/trainer, and then carry out the protocol with no supervision. An experienced supervisor determines when a technician is adequately trained since there is no precise level of training that can be measured. Once the technician demonstrates competence in executing all the aspects of the *in vitro* NRU cytotoxicity test method(s), it is appropriate to initiate routine assessments of observations among personnel using a benchmark control test substance (SLS for these two NRU test methods) to ensure consistency.

The laboratories in this study were experienced in performing *in vitro* cytotoxicity assays but were required to train and develop additional skills through Phases I and II (e.g., data collection and transfer to Excel® and PRISM® templates). Inexperienced laboratory personnel were trained by completion of "training" NRU assays using SLS. In the early phases of the ICCVAM/ECVAM validation study, the laboratories continued training by the testing of coded reference chemicals of various toxicities and performing solubility testing on the chemicals. This training improved proficiency among the staff of the laboratories for the final phase of the validation study.

GLP-Compliance Proficiency Criteria

ECBC and IIVS conducted this study in compliance with GLP Standards (see **Section 8.1.1**). The appropriate QA unit (as per GLPs) reviewed the various aspects of the study and issued a QA statement that identified whether the methods and the results described in the Final Report accurately followed the test method protocol and reflected the raw data produced during the study, respectively, and provided assurance that all testing was done under the principles of GLP. FAL (non GLP-adherent) followed GLP standards referenced in **Section**

8.1.1 as guidelines for conducting this study. FAL had no QA unit to judge their compliance with GLP guidelines.

11.3 Test Method Cost Considerations

11.3.1 3T3 and NHK NRU Test Methods

Laboratory Costs

Supplies such as cell culture chemicals, the reagents used to measure NRU, and cell culture plasticware are available from numerous suppliers and are not cost prohibitive. Major instruments and equipment that *in vitro* cytotoxicity laboratories need to implement the *in vitro* NRU cytotoxicity test methods are described in **Section 11.1.1**.

The 3T3 NRU test method is generally less expensive to use than the NHK NRU test method. One vial of the immortalized 3T3 cells ($180) can be propagated indefinitely by passaging cells and periodically cryopreserving pools (i.e., numerous vials of cells). NHK cells require a fresh sample of primary cells for each test run ($380 per vial). Since primary NHK cells are only passaged once after initiating into culture, there are no cells available to cryopreserve a stock pool of cells. The D-MEM medium used for the 3T3 cells is less expensive, more "generic", and more readily available than keratinocyte-specific medium. (See **Table 11-1**)

Table 11-1 Costs for Cell Culture Materials and Commercial Laboratory *In Vitro* Cytotoxicity Testing

Item	Cost (approximate)	Number of Tests Possible	Other
3T3 Cells	$180/vial[1]	indefinite	One vial can produce an indefinite supply of cells by propagating the cells in culture and periodically freezing a pool of cells.
NHK Cells	$380/vial[1]	~5 (96-well plates)	Since cells are passaged only once beyond cryopreservation, new ampules should be thawed frequently to maintain continuous testing.
Dulbeccos' Minimum Essential Medium (D-MEM) with supplements	$20/500mL[1]	~15 (96-well plates)	Establish cells in culture (~20 mL/vial of cells; 60 mL/3 vials), seed cells in 96-well plates (12 mL/plate; 180 mL/15 plates); prepare stock solution and eight concentration dilutions (~20 mL/chemical; 300 mL/15 plates).
NHK Medium with supplements	$80/500 mL[1]	~15 (96-well plates)	Same as DMEM (above)
Commercial Laboratory Testing (MB Research Laboratories)	$1050/$1950 (USP/ISO) per 3 test materials[2]	1 test/material	*in vitro* NRU cytotoxicity test (24-hour test period)
Commercial Laboratory Testing (Institute for In Vitro Sciences))	$1120 (GLP) per test material (minimum of 5 materials)[2]	1 range finder, 2 definitive tests per test material	*in vitro* NRU cytotoxicity test (48-hour test period)
Commercial Laboratory Testing (Institute for In Vitro Sciences))	$1850 (GLP) per single test material[2]	1 range finder, 2 definitive tests per test material	*in vitro* NRU cytotoxicity test (48-hour test period)

[1]catalogue price
[2]personal communication

Commercial Testing Laboratories

A representative of MB Research Laboratories (Spinnerstown, PA, http://www.mbresearch.com/) provided a quote (personal communication 2005) for an *in vitro* NRU cytotoxicity test (24-hour [and not a 48-hour] test period) of $1050/$1950 (USP/ISO) per set of three test chemicals. The lead laboratory for the NICEATM/ECVAM study, IIVS (Gaithersburg, MD, http://www.iivs.org/) provides commercial laboratory GLP-compliant testing using this study's protocols (48-hour test period) at a cost of $1120 - $1850 per chemical/sample (personal communication with Hans Raabe [IIVS] 2005).

11.3.2 *In Vivo* Rodent Acute Oral Toxicity Testing

Table 11-2 provides commercial prices for acute oral systemic toxicity testing.

316
317 MB Research Laboratories performs the UDP test at a cost of $750 for three rats and charges
318 $250 for each additional rat needed. In the best-case scenario, the UDP test needs only three
319 rats ($750). In the worst-case scenario, this test would need an additional 12 rats (15
320 maximum for the test); the total cost of the test would be $3750. In this costing strategy,
321 $250 is saved from the total cost of the UDP for each rat saved by using the 3T3 or NHK
322 NRU test method to predict the starting dose. Considering that adding the *in vitro* NRU
323 cytotoxicity test costs from $350 to $1850 per chemical, the NRU test does not provide cost
324 savings if fewer than two to six animals are saved.
325
326 The President of Product Safety Laboratories (Dayton, NJ,
327 http://www.productsafetylabs.com/), Gary Wnorowski, provided a cost quote of $2700 for
328 determination of an LD_{50} value using the UDP test; the cost is independent of the number of
329 rats that are needed. Each testing dose is administered ~24-48 hours after the previous dose
330 and each animal test generally does not exceed four days. Time involved in providing the
331 LD_{50} value is approximately three months (initiation of the test to provision of the final
332 report). Knowing the estimated LD_{50} value does not affect the cost of the *in vivo* test in this
333 case but could reduce the number of animals needed for the test.
334
335 Bio Research Laboratories (BRL) performs the Acute Oral Rat Toxicity Test bioassay to
336 determine the relative acute toxicity of an unknown substance. The method determines
337 lethality and signs of acute toxicity from a waste sample administered in a single dose by
338 gavage to a limited number of rats. The bioassay determines if the test sample exhibits a
339 median lethal dose (LD_{50}) either greater than or less than a regulatory threshold
340 corresponding to a hazardous waste designation (i.e., 5000, 500, 50 mg/kg). A minimum of
341 ten rats is used at the tested dosage for the pertinent regulatory threshold value that is
342 relevant to the test sponsor. Knowledge of the estimated LD_{50} does not reduce animal use or
343 test costs if a single predetermined dose is tested.
344
345

Table 11-2 Commercial Prices for Conducting *In Vivo* Acute Toxicity Testing

Test	GLP-Compliant	Non GLP-Compliant	Company
Acute Oral Toxicity UDP: Limit Test - 2000 mg/kg	$1200	$1000	Product Safety Laboratories (PSL)
Acute Oral Toxicity UDP: Limit Test - 5000 mg/kg	$800	$650	PSL
Acute Oral Toxicity UDP: LD_{50}	$2700	$2200	PSL[a]
Acute Oral Rat Toxicity: single dose[b]	$950	NA	Bio Research Laboratories (BRL)
Acute Oral Rat Toxicity: two doses[b]	$1500	NA	BRL
Acute Oral Rat Toxicity: LD_{50}	$3000	NA	BRL
Acute Oral Toxicity – UDP	$730 for the first 3 animals; $250 each additional animal	NA	MB Research Laboratories[a]

[a]provided to NICEATM through personal communication

[b]Washington State Biological Testing Methods #80-12 For the Designation of Dangerous Waste; Part B: Acute Oral Rat Toxicity Test [http://www.ecy.wa.gov/pubs/80012.pdf] The method is an adaptation of the EPA Health Affects Test Guidelines OPPTS 870.110 Acute Oral Toxicity and American Society for Testing and Materials (ASTM) methods E 1163-90 (Standard test method for estimating acute oral toxicity in rats) and E 1372-90 (Standard test method for conducting a 90-day oral toxicity study in rats).

11.4 Time Considerations for the 3T3 and NHK NRU Test Methods

The 3T3 NRU Test Method

Approximately one week is needed to thaw cryopreserved 3T3 cells, propagate the cells in flasks, and passage/subculture the cells at least two times before subculturing to the 96-well test plate. After subculture into 96-well plates, the cells are incubated another 24 hours to reach the proper percentage of confluency, and then exposed to test chemical for 48 hours. The entire 3T3 NRU assay process takes approximately 10 days. However, once the cells are established in culture, they can be passaged for approximately two months before starting the initial propagation from frozen stock. Multiple chemicals can be tested at the same time, and different tests can overlap each other; thus, many chemicals can be tested in a relatively short time.

The NHK NRU Test Method

Approximately one week is needed to thaw cryopreserved NHK cells, propagate the cells in flasks, and passage/subculture the cells (once) directly to the 96-well test plate. After subculture into 96-well plates, the cells are incubated another 48-72 hours to reach the proper

percentage of confluence and then exposed to test chemical for 48 hours. The entire NHK NRU assay process (range finder or definitive test) requires approximately 11-12 days. Cells can be seeded at different densities in the culture flasks so that passaging the cultures can take place on different days. Once the cells are established in culture, they are passaged once to the 96-well test plates. Multiple chemicals can be tested at the same time, and different tests can overlap each other; thus, many chemicals can be tested in a relatively short time.

In Vivo Testing

According to guidelines for acute oral toxicity testing for the main test and limit dose test, single animals or groups of animals are dosed in sequence, usually at 2-4 day intervals, and observations are generally made for up to 14 days (for animals that are not moribund) (EPA 2002a; OECD 2001a; OECD 2001b, OECD 2001c). The addition of NRU testing to estimate a starting dose prior to the implementation of the UDP main test or limit dose test will take 10-12 days, but could save up to 14 days of observation for every animal saved.

11.5 Summary

- All equipment and supplies are readily available. Direct communication with the NHK medium supplier assured that specific lots of medium were available to the laboratories. The test methods should be easily transferable to laboratories experienced with mammalian cell culture methods.
- Much of the training and expertise needed to perform the 3T3 and NHK NRU test methods are common to all mammalian cell culturists. Additional technical training would not be intensive since there are no extraordinary techniques needed and these test methods are similar in general performance to other *in vitro* mammalian cell culture assays. GLP training should be provided to technicians to ensure proper adherence to protocols and documentation procedures.
- Price levels for commercial testing for one chemical are $1120 to $1850 (**Table 11-2**) for *in vitro* NRU cytotoxicity testing to determine the IC_{50} (IIVS, personal communication) versus $750 - $3750 (**Table 11-2**) for *in vivo* rat acute oral testing for LD_{50} determination. Comparison of costs of the *in vitro* testing to *in*

401 *vivo* testing is difficult since the *in vitro* NRU cytotoxicity test methods are not
402 replacements for the animal testing. Use of these test methods may not
403 necessarily reduce the overall cost of the *in vivo* rat acute oral toxicity test but
404 can reduce the number of animals needed for a study.
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407